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by

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CONTENTS

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S.R.W.

I. INTRODUCTION

The inhibitory effect of some anesthetic agents on monosaccharide uptake in human erythrocytes has been demonstrated by Greene (21). The study here reported demonstrates that one of these gases, halothane, is taken up less rapidly by galactose-loaded than by control erythrocytes. Galactose is believed to pass across red cell membranes by a carrier mechanism common to several monosaccharides. This study is offered as evidence that halothane may be transported across human red blood cell membranes by the monosaccharide carrier mechanism in addition to simple diffusion.

Membrane permeability (10)

Permeation of the cell membrane by water or solutes may occur in a variety of modes. The least complex of these, simple diffusion, dictates that water and solutes move through a membrane as free molecules in response to an osmotic or electrochemical gradient. Concentration, molecular or ionic size, hydration, valence, and molecular solubility in membrane lipid all influence the ability of solute particles to cross the cell membrane by simple diffusion (17). Although this mode of diffusion is independent of metabolic energy, a characteristic of erythrocyte monosaccharide transfer, it cannot be wholly responsible for sugar passage in the human red cell membrane. A number of observations support the concept of a mobile carrier molecule within the erythrocyte membrane which conveys monosaccharides from the external to the internal surface under transport rates and conditions incompatible with simple diffusion.

Carrier transfer

Fick's Principle postulates a linear relation between transport rate and concentration gradient under conditions of simple diffusion:

$$\frac{ds}{dt} = P\ (S)_e - (S)_i$$

where P is the permeability coefficient, $\frac{ds}{dt}$ the rate of forward sugar transfer, and $(S)_e$ and $(S)_i$ the extra- and intra- cellular concentrations of the sugar. If Fick's Principle is applied to monosaccharide transfer in the human erythrocyte, one finds that P is a variable rather than a constant, and is inversely proportional to the concen-

tration gradient across the membrane. At high monosaccharide concentrations transport rate reaches a maximum. In this state of "saturation kinetics" further increase in $(S)_e$ is accompanied by a decrease in the apparent P. This is presumably caused by full utilization of the carrier. Unbound sugar then becomes essentially non-penetrating.

Carriage by simple diffusion is non-competitive; that is, increases in concentration of other monosaccharides do not affect the transport rates of species under observation. Carrier transfer, also known as facilitated diffusion, is competitive; the transfer rate for a given monosaccharide decreases in the presence of any of a number of sterically similar sugars which compete for the carrier.

A further characteristic of simple diffusion is that elevation of temperature produces relatively little change in transfer rate. The exact ratio by which the velocity of a reaction changes when subjected to a ten-degree centigrade rise in temperature is referred to as the temperature coefficient or "Q_{10}." While this value is close to 1 for the diffusion of small molecules through inert membranes, it approaches or exceeds 2 for enzyme- or carrier- dependent transfer through biologically active membranes. The Q_{10} for glucose penetration into erythrocytes has been calculated at from 2.5 to 6.25 by the investigators reviewed by Cronau (8).

The observation that monosaccharides may cross the erythrocyte membrane against a concentration gradient is the most conclusive demonstration of the inadequacy of simple diffusionto explain sugar transport. In addition, both this uphill transfer and diffusion along concentration gradients occur without the expenditure of metabolic energy. Inhibition of glycolysis by ouabain has no effect on monosaccharide transfer.

Widdas (69) and Rosenberg and Wilbrandt (58)developed kinetic proofs that the energy required for "uphill transport" could be dervived from the simultaneous "downhill" movement of a second substrate, sharing the same carrier but moving across the membrane in the opposite direction. This has since been accepted as a characteristic of the carrier system; such "counter-flow" has been shown even in the absence of "uphill" transport.

The monosaccharide carrier system

Widdas (69) notes that Kozawa had observed in 1914 that glucose penetrated erythrocytes rapidly, while methyl glucoside was non-penetrating. Ege ( ) observed that the rate of penetration of glucose was reduced at high concentrations. Wilbrandt( ) found rapid penetration of D-xylose and L-arabinose but little penetration by the stereoisomers L-xylose and D-arabinose. The rapidly transferred species were also mutually competitive inhibitors. Similar studies by Le Fevre and Davies demonstrated competitive inhibition among sterically similar monosaccharides but demonstrated that sorbose and fructose entered only by simple diffusion kinetics( ). Widdas found that fructose and sorbose traversed the erythrocyte membrane by simple-diffusion kinetics, but observed inhibition of this ketose transfer by glucose ( ). Earlier studies ( ) had demonstrated variation of the permeability coefficient of glucose in a manner consistent with saturation of a biologic carrier( ). Joanny, Corriol, and Kleinzeller ( ) noted facilitated diffusion in in vitro preparations ofguinea pig cerebral cortex, and demonstrated saturation kinetics, competitive inhibition, and stereospecificity similar to that seen in primate erythrocytes. Joanny and Corriol ( ) were unable to demonstrate inhibition of this process by ouabain. Facilitated diffusion of glucose is also

present in dog and rat brain tissue(15)(37).

In other biologic systems, monosaccharide carrier systems have been found in the rat diaphragm(44),the isolated perfused rat heart (44), the sheep placenta (68), erythrocytes of the fetal guinea pig and other non-primate fetuses (11), and the Erlich ascites tumor cell (32). Studies of the intestinal mucosa of the dogfish shark by Read (56) demonstrated inhibition of cycloleucine transport by either preincubation or simultaneous ten-minute incubation with galactose, although this carrier system is evidently energy-dependent. Gilbert has found transport, possibly active, of xylose against a concentration gradient in the rat (19) and guinea pig (18) brain. Active transport of glucose or other monosaccharides has been demonstrated in the guinea pig intestine (51) and active reabsorption of some sugars occurs in renal tubules (63). Kotyk and Michaljanicova (34) andKotyk and Haskovec (33) have shown that Saccharomyces cerevisiae (baker's yeast) contains two constitutive monosaccharide carriers and a third (inducible) carrier for galactose. Steveninck and Dawson (66) have also found two modes of galactose transfer in baker's yeast. They state that in uninduced cells transfer is mediated passively via a carrier and is not pH-dependent; induced cells demonstrated highly pH-dependent active transport which may share the same carrier.

Monosaccharide transfer across the erythrocyte membrane is inhibited by a variety of compounds. Dinitrophenol, dinitrofluorobenzene, thymol, phloretin, phenolphthalien, and stilbesterol are demonstrated inhibitors (16, 39); their reversibility has not been demonstrated. Mercuric chloride and p-chloromercuribenzoate inhibit glucose transfer by combining with sulfhydryl groups. Glucose transfer

from the cytoplasm to the surrounding medium is also impaired by these agents. The addition of cysteine reverses inhibition by mercurials.

Hunter (29) describes butanol inhibition of monosaccharide transfer, but this was not confirmed by Greene and Cervenko (22) using methods similar to those employed in the present study. Hagland (24) has shown that butanol increases the rates of permeation of coelomic eggs of Rana temperia by water; as the minimum toxic concentration of butanol was approached, the permeability coefficient for water approached infinity, suggesting the elimination of a diffusion barrier. This suggests that osmotic phenomena in the presence of butanol may not be a reliable indication of carrier activity. Copper ion has produced inhibition of carrier transfer of glycerol (72). Oski, Root, and Winegrad (46) found erythrocyte glucose consumption unaffected by the presence of insulin, but demonstrated a 32.9 to 59 per cent decrease in the presence of purified human growth hormone at a concentration of 16 ug/ml. Fructose consumption fell 44.7% under similar conditions. Carbon dioxide consumption remained constant despite inhibition of uptake, and the concentration of fructose-6-phosphate and glucose-6-phosphate underwent identical rises in both experimental and control preparations. This suggests that alteration of monosaccharide transfer in erythrocytes has little if any effect on the rate of monosaccharide metabolism.

Insulin increases transport capacity for glucose thirteenfold in the isolated perfused rat heart, in which membrane kinetics are the same as in erythrocytes, but this is reflected by increased phosphorylation ($_5$0). In preparations of eviscerated, nephrectomized dogs, Levine, Goldstein, Huddlestun and Klein (40) demonstrated an insulin-stimulated increase in galactose uptake, despite the fact

that no transformation of galactose appeared in tissue. The dog erythrocyte is impermeable to galactose, however. Morgan, Regen and Park (44) have noted counterflow of membranal glucose in the presence of extracellular 3-0-methylglucose in the rat diaphragm, and have noted acceleration of the same phenomenon in isolated perfused heart muscle treated with insulin. They suggest that insulin accelerates transfer by the mobile membrane carrier. Langdon and Sloan (35) state that insulin increases the number of specific sugar-binding sited in the rat ependymal fat cell.

Carbon dioxide increases the rate of glucose transfer in human erythrocytes. Greene (21) has theorized that carbon dioxid e might increase the number of operable carrier sites or act to increase the affinity for the carrier at the external membrane surface; it might alternatively, he states, increase the rate of dissociation of glucose-carrier complexes at the inner surface of the membrane.

LeFevre (38) has postulated an upper limit of 500,000 carrier sites per erythrocyte membrane, but Stein has calculated that one million sites are present on the basis of the number of dinitrophenyl residues necessary to complately label the carrier (65).

Membrane Kinetics

Lineweaver and Burk (41) presented graphic adaptations of the Michaelis-Menten equation to various equilibria attainable in enzymic systems. The substitution of carrier rather than enzyme molecules does not change the Lineweaver-Burk derivations, and they have been used to characterize the monosaccharide transport system.

Those cases considered in monosaccharide transfer studies

are summarized below, the original case numbers of Lineweaver and Burk, but with the substituion of a carrier for an enzyme.

The original Michaelis-menten equation is:

$$v = v_{max}(S)/ (K_s+(S))$$

where: v = the velocity of the reaction

v_{max} = the maximal velocity attainable by the reaction.

(S) = substrate concentration (in this case, of monosaccharide)

K_s = the substrate concentration which produces half-maximal velocity (Michaelis constant); This also the dissociation constant:

$$K_s = \frac{(C)(S)}{(CS)}$$

Carrier Concentration

Case I.

$C + S \rightleftarrows CS$ (where C represents one carrier molecule, and S one substrate molecule.)

$$1/V = K_s/ V_{max}(S) + 1/V_{max}$$

This equation applies in the case that one carrier molecule accepts one sugar molecule and the complex is the active species. The plot of I/v against I/(S) is a straight line. The ordinate intercept is I/V_{max}, and the slope is K_s/V_{max}.

Case II.

$C + nS \rightleftarrows CS_n$

$$v = V_{max}(S)^n/ (K_s + (S)^n)$$

In this case, one or more sugar molecules are bound to each carrier. A plot of I/v against $(S)^n$ yeilds a straight line. The ordinate intercept and slope have the same significance as in Case I, but K_s is now the nth power of the substrate at half-maximal velocity. This equation only applies when the carrier binds a constant number of substrate molecules.

See Case V.

In cases in which each component of the ratio $(CS_n)/(C)$ can be evaluated experimentally, the equation:

$$(CS_n)/(C) = (C)^n/K_s$$

may be translated to linear form by taking the log of both sides. The slope of the plot of log $(CS_n)/(C)$ against log (S) is the value of n, equal to unity in the simplest case, and the intercept at S = 1 is the log $1/K_s$.

In that of the red cell membrane, the observed velocity is the difference between the inward and the outward transfer of monosaccharide.

$$v = V_{max}\left[(S_o)^n/\ (K_s\ \ (S_.)^n)\right] -- V_{max}\left[(S_i)^n/(K_s\ (S_i)^n)\right]$$

$$v = V_{max}\left[\frac{(S_o)^n}{K_s\ \ (S_o)^n} \quad -- \quad \frac{(S_i)^n}{K_s\ \ \ (S_i)^n}\right]$$

Employment of the "relative substrate concentration" defined as the ratio of the substrate to the dissociation constant on each side of the membrane enables the following substitution;

$$(S'_x)^n = (S_x)^n/K_s$$

$$v = V_{max}\left[\frac{(S_o')^N}{(S_o')^n} \quad -- \quad \frac{(S_i')^n}{(S_i')^n I}\right]$$

V_{max} may be thought of as a consequence of two factors, the capacity of the membrane carrier, and its state of saturation. The carrier saturation factor is the difference between two degrees

of saturation, possible in the system; I, indicating that the carrier is fully saturated, and O, indicating that it is not fully saturated. Increasing the number of sugar moieties carried from monocomplex to dicomplex has no influence on the V_{max} because it is predicated experimentally upon full saturation.

The capacity of the membrane carrier is determined by the permeability constant and the total carrier available, C_t. The relationships which obtain between these factors are:

Capacity = (permeability constant) x (C_t)

Saturation factor = 1

V_{max} = s.f. x capacity = capacity

under the condition that the diffusion constant of the free carrier is equal to the carrier - sugar complex.

CASE IV

$$2C + S + I \rightleftharpoons CS \text{ (active)} + CI \text{ (inactive)}$$

$$I/v = (I/V_{max}) \left[(K_s K_{s_{K_I}} (I) \right] \left[I/(S) \quad I/V_{max} \right]$$

This describes both competitive and non competitive inhibition. In each case the plot I/V against I/(S) is a straight line. Comparing this to the straight line form equation y=mx+b one notes that the term $K_s(I)/K_I$ represents as additive increase in the slope; when this term does increase the slope, V_{max} is unchanged. This is Evidence of noncompetitive inhibition.

Competitive inhibition decreases the V_{max} directly by lowering the number of sites available to substrate. The slope is unchanged, but the ordinate intercept is higher.

Case VII.

$S \longrightarrow S'$,(steady state(S')), S' C CS

Where:

$S \rightarrow S'$ represents rate-limiting diffusion with a velocity constant of k_I

$$v = V_{max} k_i(S) \left[V_{max} k_I(S) - v \right]$$

This applies in the case of a rate-limiting step preceding the carrier-sugar stage. This limit may be that of diffusion or of a chemical process.

Although the rate-limiting step in the monosaccharide transfer system has usually been characterized as the formation or diffusion of the sugar-carrier complex, there is no assurance that the separation of the carrier complex from the aqueous lipid interference is not more difficult.

Membrane kinetics - II

Definition of the kinetics of glucose transfer based on the assumption that the glucose-carrier complex crossed the erythrocyte membrane slowly were developed by Widdas.(68) Later Rosenberg and Wilbrandt (58) developed kinetics applicable to systems with rate-limiting steps. Each of these was based on the assumption that the sugar-carrier complex was a monocomplex, that is, that each carrier molecule bound only one sugar molecule. Subsequent observations of glycerol transport and transport of pairs of different monosaccharides by Stein (64.65) suggested dimer formation by enzymatic action or dicomplexes in which the carrier molecule bound two sugar moieties. Wilbrandt and Kotyk (71) confirmed Stein's postulate using nonisotopic sugars added to erythrocyte preparations previously incubated with isotopic solutions of the same monosaccharides. Cronau (8) found that for all theoretical cases of the dicomplex carrier system, the kinetics of the initial rate calculated could be approximated by assuming a first-order process as in the monocomplex carrier system. Miller has since proposed the existence of either pores or a membrane flanked on each side by a first-order slow diffusion layer. (52) Weinstein and Koo (67) have shown particles extending completely through, and associated with discontinuities of, the erythrocyte membrane.

The most widely held and most completely investigated theory continues to be that of a mobile carrier which probably binds two molecules of monosaccharides. Predominance of dicomplexes may be due to either higher mobility or preferential formation.

Molecular binding of sugars

Bobinski and Stein (4), working with Bonsall and Hunt, (5) have prepared chromatographic columns in which the stationary

phase is composed of ghost erythrocyte membranes. These were adsorbed onto Celite or DEAE-cellulose and displayed the monosaccharide-transfer properties of erythrocytes. Transport of sugars on these columns was inhibited by phloretin and dinitrofluorobenzene. Elution of these columns with a linear gradient of half-molar sodium phosphate at pH 7 yielded five major peaks. The second of these peaks was extracted by molar sodium iodide from ghosts and was associated with 60% of the retardation of glucose flow on the membrane-coated columns. Langdon and Sloan (11) found that imine bonds formed between monosaccharides and the lysyl residues of human erythrocyte membrane proteins. They claimed that the dissociation constants of these sugar-protein-imine complexes resembled those proposed for sugar-carrier complexes. They also found competitive inhibition by various monosaccharides and other competitive inhibitors and suggested that the lysyl-bearing residues were monosaccharide carriers. LeFevre (36) disputes this on kinetic grounds, arguing that carrier sites of lower affinity would better account for the data.

LeFevre, Jung and Chaney (38) constructed a three-layer model of the erythrocyte membrane using a U-tube. The lower portion of the tube contained chloroform; aqueous phases representing the internal and external cellular fluids were placed in the U-tube arms. They found that passage of glucose across the chloroform phase was markedly accelerated by the addition of any of a variety of phospholipids but that the aqueous-membrane interfaces presented the major barrier to diffusion. At high glucose concentrations the rate of transfer increased, suggesting dimerization. A crude lipid extract had greater affinityfor ribose than glucose and transported inositol as well. These characteristics are not those of the erythrocyte carrier, which transports glucose better than ribose and does not transport inositol.

They suggest that study of the purified fractions may disclose different properties. In the same paper, the suggestion is made that erythrocyte monosaccharide transfer may involve the serial participation of two membrane elements:

Sugar + Surface reacting site (? protein) → Surface complex + Phospholipids → Mobile complex (?phospholipid-sugar-protein)

A similar dissociation would occur at the inner membrane surface.

Anraku (1, 2) has isolated a galactose-binding protein from galactokinase-lacking mutants of E. coli K 12; these binding proteins have a molecular weight of 35,000, and complex with substrate in a molar ratio of 1:1. Since the rate of galactose uptake exceeds that explained by stoichiometric bonding, turnover capability must exist for the carrier.

Kotyk and Michaljanicova (34) have isolated a monosaccharide-binding protein fom Saccharomyces cerevisiae which is present in the internal cellular fluid, but has no activity in membrane transport. They caution that this makes the study of kinetic data difficult. Although no similar study of the erythrocyte has been located, the kinetics of red cell ghost membranes are those of the intact cell.

Anesthetics and the erythrocyte membrane

Anesthetics exert two distinct, although possibly related, effects on the erythrocyte membrane. The first of these is stabilization of the membrane against hemolysis (6). Seeman (61) found that thirteen aromatic and ten aliphatic alcohols, all credited with anesthetic action, stabilized the erythrocyte against hypotonic hemolysis at low concentrations, but caused hemol-

ysis at higher concentrations. Nine steroids did likewise. Furthermore, the concentration of alcohol which caused fifty per cent stabilization was that found to be the minimum anesthetic concentration (AD_{50}) by Meyer and Hemmi (42), using a variey of alcohols on tadpoles, and Skou (62) , using alcohols, thymol, menthol, or beta-naphthol on frog sciatic nerve. The amount of membrane stabilization occuring at a steroid concentration of 7×10^{-5}M correlated with the AD_{50} for steroid anesthesia in rats found by P'an and Laubach (47). Although high concentrations of tertiary-amine local anesthetics and alcohols cause depolarization of nerve fiber, depolarization is found at the AD_{50} for propyl alcohol only and is not necessary to a blocking effect.

Skou also demonstrated a close parallel between the blocking potencies of both tertiary amines and alcohols and their ability to penetrate a monolayer of frog sciatic nerve lipids extracted with benzene (62). Jacobs, Glassman, and Parpart (30) found in 1935 that carbon dioxide stabilized the human and rat erythrocyte membranes against temperature-induced hemolysis in solutions of isotonic glycerol. This was accompanied by an increase in magnitude of the temperature coefficient (Q_{10}) of glycerol entrance.

Rand (55) demonstrated that hemolysis follows upon application of strain, such as increase in surface area, rather than of stress, such as bending forces.

Greene and Cervenko (22) demonstrated a stimulatory effect of carbon dioxide on glucose transfer in human red blood cells at 21-23°C. Mercuric chloride inhibited the stimulatory effect of carbon dioxide, but not to as great an extent as in control preparations not treated with carbon dioxide. Neither ouabain nor butanol influenced glucose transfer in control or carbon dioxide treated red cells. The anesthetic gases halothane, methoxyflurane, and diethyl ether depressed, but did not eliminate, the acceleration produced by carbon dioxide.

Enns demonstrated enhancement of labelled carbon dioxide transfer across ghost erythrocyte membranes by bicarbonate ion (14). This enhancement was inhibited by the carbonic anhydrase inhibitor acetazolimide in red cell ghosts, but not in whole blood.

Studies by Greene (21) on the effect of both halothane and methoxyflurane on the transfer of a variety of monosaccharides demonstrated a depressant effect on the transfer of L-sorbose, D-ribose, D-arabinose, and D-galactose, but had no significant effect on the transfer of D-fructose, L-arabinose, D-xylose, or D-glucose. Nitrous oxide also decreased galactose transfer, while diethyl ether had no effect on galactose uptake. The effects of the latter three gases on transport of other sugars were variable.

Wilbrandt and Rosenberg (70) have distinguished between penetrating and non-penetrating inhibitors of transfer systems. In the presence of a non-penetrating inhibitor the competition at the external surface is the determinant of inhibition. However, in the presence of a competitive inhibitor which is penetrating, the kinetics when the inhibitor (I) is present in equal concentrations on each side of the membrane are:

$$v = V_{max} \frac{(S_o')}{(S_o^*)\ (I_o')\ 1} \quad \frac{(S_1')}{(S_1')\ (I_1')\ 1}$$

In this case the anesthetic may act as a competitive inhibitor at high concentration or may act to increase transfer of S_o via the counterflow system previously discussed. Thus, the action of a particular anesthetic upon monosaccharide transfer depends not only upon the ease with which it penetrates the lipid membrane as a simply-diffusing solute, but also upon its affinity for the carrier molecule, the ultimate concentration of anesthetic on either side of the membrane and the concentration gradient of the monosaccharide under study.

Halothane

Halothane anesthetic gas has been characterized by several investigators(59) and the pertinent physical and chemical characteristics are summarized in Table 1.

Commercial halothane contains thymol in low concentration. Halothane reacts with rubber and some plastics, but not with plyethylene. Although sixteen trace contaminants were noted by Chapman et al., none was present in a concentration of over 12 ppm. (7). At high temperatures in the presence of copper, halothane may polymerize to form the cellulotoxic compound 2,3-dichloro-1,1,1,4, 4,4 hexafluorobut-2-ene. Halothane is extremely soluble in lipid as is methoxyflurane (63) but Greene found halothane to be more inhibitory toward galactose transferthan was methoxyflurane. Lipid solubility alone, therefore, cannot account for this inhibition.

Hans and Helrich (25) have reported the effect of temperature on the solubility of halothane in human blood and in brain tissue homogenates. In human brain gray matter the tissue/air partition coefficient of halothane increases in a curvilinear manner as temperature decreases from normal to below 28°C. In human whole blood at 38°C and at a hematocrit of 50%, 70% of the halothane is carried by plasma and 30% by blood cells. If both hematocrit and hemoglobin concentrations are increased the partition coefficient decreases slightly, due to the greater solubility of halothane in plasma than in red cells. If the hemoglobin concentration is increased independently of hematocrit, the partition coefficient increases, suggesting hemoglobin binding of halothane. Twelve to twenty per dent of halothane admin-stered to humans as anesthesia is metabolized (57).

Scholander (60) and others (13, 27, 28) have demonstrated facilitated diffusion of oxygen through hemoglobin-saturated Milipore filters. This occurs after full saturation of hemoglobin (that is, total conversion to oxy-hemoglobin) and may be due to exchange of

oxygen moleculesby collision of hemoglobin molecules. However, oxygen crosses the erythrocyte membrane by simple diffusion only.

III. MATERIALS AND METHODS

Principle

Fresh human packed red cells were exposed to identical atmospheres of room air during a one-hour incubation with either galactose in phosphate buffer, or phosphate buffer only. The phosphate buffer was chosen for its support of monosaccharide utilization (57). They were then exposed to identical concentrations of halothane in an instrument designed to indicate the difference in volumes of halothane taken up by the control and galactose-treated red cells. Serial determinations of the hematocrits in each of these solutions and in similar suspensions not exposed to halothane were made and these were accepted as an osmotic index of galactose penetration into red blood cells (38, 39, 71). The supernatant was observed at each hematocrit determination for presence of hemolysis.

Apparatus (Figure 1)

The differential manometer fabricated for these experiments was based upon that designed by Winterstein in 1912 (72). A horizontal glass tube serves as the manometer proper. At each end the manometer is joined to glass chambers of identical shape and volume by polyethylene tubing and metal cannulae. The bore of the manometer tube is lubricated with a soap suspension. A small column of this suspension moves freely in response to pressure changes at either end. Three-way stopcocks betw-en the manometer and the tubing allow connection between the glass chambers *via* the manometer; this is the configuration maintained during measurement of pressure changes in the flasks relative to each other. One may also independently

vent either chamber to the atmosphere, either directly or through the manometer. Each chamber is capped by a polyethylene stopper pierced by metal cannulae which allow access for the purposes of displacing the chamber gas by that under study and of placing cell suspensions into the bottom of the chamber. The stoppers are sealed completely with Leak-Lock*, a plastic paste sealer, and the stopcocks are lubricated with a silicone preparation.

The glass chambers sit in a constant-temperature water bath at 27°C (± 1°C), and the remainder of the apparatus is supported conveniently above.

An atmosphere of halothane saturated at 21°C is generated by a Foregger Copper Kettle vaporizer (45). Simultaneous delivery to each flask through a Y piece ensures equal concentrations of halothane (32%) in each chamber.

Calibration of the manometer in cubic centimeters is presented in appendix 2.

Procedure

Fresh human red blood cells from the same adult male volunteer were used for all experiments. Whole blood was drawn by venipuncture into a heparinized syringe and then centrifuged in chemically clean glassware for 15 minutes at 25,000 RPM. After aspiration of the supernatant plasma and leukocytes the resulting erythrocytes were thoroughly mixed. A 2 ml. aliquot was then pipetted into each of two polyethylene syringe tubes which served as mixing and incubation chambers. 0.50 ml. of Sørenson's phosphate buffer (pH 7.38) was then added to the control aliquot (appendix 1). An equal

*A product of Highside Chemicals Inc., Clifton, N.J., which is soluble in alcohol but resistant to "Freons," methyl chloride, methylene chloride, oil, benzene, toluene, gasoline, trichoroethylene, CCl_4, formaldehyde, SO_2, and water.

volume of 1.43 M galactose in Sørenson's buffer was added to the experimental aliquot. After thorough mixing the suspensions were incubated at 15°C in air for one hour (in the case of those preparations to be exposed to halothane uptake experiments) or two hours (in the case of those experiments not exposed to halothane.)

Before the conclusion of the incubation period the atmosphere in the glass manometer chambers was displaced by a ten-minute flow of 32% halothane in compressed air. $P_{halothane}$ equalled approximately 300 mm. Hg. The control and experimental erythrocyte suspensions were then injected into opposite sides of the manometer; the chambers were then sealed from the surrounding atmosphere simultaneously and connected to the manometer tube. Differences in pressure between the flasks, and therefore halothane uptake differentials, induced movement of the soap column until pressure equalization occurred. Sequential observations of the position of the meniscus of a small soap column were made.

Separate experiments, which will be termed "blanks" for clarity, were performed with the same apparatus using red-cell-free mixtures of water (distilled, ion-free) and either buffer or buffered galactose. The volumes of the buffers and the concentration of the galactose in the sugar buffer were identical to those stated for the set of experiments containing red cells. The water volume was 2.0 cc., equal to that of the packed cells. Serial determinations of the microhematocrit were performed by the Strumia micro method used by Greene. These were determined in packed cells before the addition of buffer, and at 5, 60, and 120 minutes after beginning incubation with buffer and buffered galactose.

At the conclusion of the experiment the manometer was opened to the room atmosphere and observed for vigorous movement of the meniscus away from the opened vent. This was accepted as proof that the internal pressure of the system was sub-

atmospheric. Careful attention was paid to maintaining an air-tight system, and each experiment was preceded by demonstration of the ability of each chamber to resist a negative pressure of 0.66 atm., approximately equal to that which would remain if all halothane was absorbed. The stopcock was occluded, and a negative pressure was applied to the flask inlet using a 3 ml. displacement of an airtight ground-glass syringe plunger. Upon release the plunger returned to its original position. Occasionally the system was subjected to a 60 mm. positive pressure by similar methods. The manometer tube joints were examined occasionally by the same method.

Measurement

1. The volume difference of halothane uptake was taken as the displacement of the meniscus from its original position and represented:

Vol. = Volume consumption by the galactose preparation. − Volume consumption by the control preparation.

Blanks were treated similarly. The volume difference was plotted against time of exposure to halothane on a linear graph.

2. The hematocrit was observed as described and the mean hematocrits compared for each of the following four sets:

1. incubated in galactose exposed to halothane.
2. incubated in buffer exposed to halothane-
3. incubated in galatose not exposed to halothane.
4. incubated in buffer not exposed to halothane.

IV. RESULTS

Halothane uptake

The uptake of halothane by both erythrocytes and red-cell-free buffer solutions was determined. Figure 3 presents the results of five independent experiments to determine the volume difference between uptake in galactose-loaded cells and uptake in cells exposed to plain buffer. Figure 4 presents the results of two independent experiments to determine the difference between halothane uptake in a mixture of plain buffer and water and uptake in galactose-loaded buffer and water. For convenience, falling lines indicate greater uptake by the solutionsnot containing galactose, and this is correlated with shift of the manometer meniscus in a negative direction as seen in Figure 1.

The "blank" experiment, using red-cell-free buffer and sugar, indicated that galactose alone did not inhibit the uptake of halothane in aqueous solution. Indeed, the volume of halothane absorbed into galactose solution within twenty minutes was in one case (c-1) over 0.11 cc. greater than control, while in the other case it was 0.05 cc. greater than control.

Uptake of halothane by erythrocytes contrasted markedly to that by cell-free solutions. Galactose-laden red cells cosistenly took up less halothane than galactose-free cells. The volume difference ranged between 0.04 cc. (case 4) and 0.11 cc. (case 1) after twenty minutes exposure to halothane. While the rate of increase in volume difference appears constant for the first ten to twenty minutes, a plateau is reached between fifteen (case 5) and fifty (case 2) minutes, indicating that uptake has become equal in both galactose-laden and

galactose-free suspensions. Two curves (cases 3 and 5) show a trend toward reversal of the volume difference after forty to fifty minutes of exposure to halothane.

It is of interest that three of the five curves in Figure 3 are nearly parallel throughout the first thirty minutes of exposure.

Galactose uptake

The mean hematocrits of red cell preparations treated as described are shown in Table 2 and more complete data are found in Appendix 3.

The immediate osmotic effect of the high extracellular galactose concentration is easily seen in both dets exposed to galactose (#'s 1 and 3). These cells occupy less than 0.8 of the volume they would had they not lost water.

No difference is seen between the sets of galactose-loaded cells at the end of one hour. Gradual uptake of galactose and associated recovery of the water flux is seen in both sets. Exposure to halothane for one hour beyond this time had no effect on galactose transfer.

The sets of erythrocytes placed in phosphate buffer without sugar show a slight, (3-4%), but definite increase over the estimated red-cell volume 5 minutes after incubation. The phosphate buffer used was itself hypotonic (154 u osm./L.) and would be expected to cause osmotic swelling of the erythrocytes. The apparent decrease in the hematocrit at 60 and 120 minutes is not significant.

Figure 2 shows each of these hematocrits as a percentage of the estimated red-cell volume. Entrance of galactose into the cell is indicated by the hematocrit change, and this is shown as a linear function throught the period observed, indicating saturation of the membrane carrier.

Hemolysis

Hemolysis was present in all cell suspensions exposed to halothane for one hour. It was not noted in any suspension before exposure, nor did it develop in any suspension not exposed to halothane, regardless of length of incubation in buffer or galactose.

V. DISCUSSION

These experiments demonstrate, in a semi-quantitative manner, that galactose may be a competitive inhibitor of halothane uptake in red-cell suspension. Since the observed differences in volume uptake of halothane were extremely small, much of the support for this argument must come from the consistency of form displayed, rather than from any statistical analysis of the data. It is obvious from the figures presented that galactose did not inhibit uptake solely because of its osmotic activity, for uptake was accelerated in cell-free galactose experiments. (This may reflect the baseline of the apparatus, rather than any halothane-binding effect of galactose, and no other conclusion may be advanced pertaining to the cell-free solutions.)

The data presented in Table 2 and Figure 2 demonstrate the deformation of red cells by osmotic dehydration and rehydration. This cannot, however, explain the findings in the halothane uptake experiments, for the red cell deforms without change in its surface area over a wide range of osmotic pressures (59), and the lipid monolayer area is not increased at low surface pressures (51). The increase in extracellular water concomitant with osmotic dehydration is also an unlikely cause of the greater uptake in galactose-loaded cells, for it was observed above that halothane uptake was not increased in aqueous solutions of galactose.

The plateau phase of the curves may be explained in two ways. Halothane uptake may be decreased in galactose-free solution if its partial pressure has fallen markedly by this time. On the other hand, processes are conceivable whereby the halothane-permeated red cell may be more permeable to further diffusion (e.g. by alteration of its lipid coating (53,65) or infrastructures).

The more attractive hypothesis is that galactose-laden red cells take up halothane at a slower rate than do normal cells, and further that the rate is dependent on the partial pressure of halothane in the atmospheric and aqueous phases above the red cells. In terms of the Lineweaver-Burk plots, the initial uptake curves of halothane in the two solutions might generate a similar curve of volume difference if they proceeded at different rates and were themselves straight lines.

The data presented in Table 2 for osmotic changes in red-cell volume differs from that of Greene in that no difference is found between the rate of galactose transfer in cells exposed to halothane and those which remain in room air. This is due to preincubation in galactose. The excess of galactose places halothane at a competitive disadvantage, while Greene accomplished the reverse with an initial excess of halothane.

VI. SUMMARY

The uptake of halothane in a suspension of red blood cells previously incubated for one hour with galactose is less than that in a native red-cell suspension in phosphate buffer. Additional experiments indicate that this is not due to the osmotic effects of the galactose molecule, which are also demonstrated.

The literature supporting a carrier-molecule theory of galactose transfer was reviewed, as was also that pertaining to the interaction of anesthetics and monosaccharides at the cell membrane. The evidence presented is advanced in support of the theory that halothane is transferred across erythrocyte membranes in part by a process competitive with that of galactose transfer.

Table 1.

Characteristics of Halothane:

Formula CF_3 CHBrCl

Boiling point 50.2°C

Vapor pressure 300 mm Hg at 20°C

Heat of vaporization 35 Cal./gm.

Partition coefficients

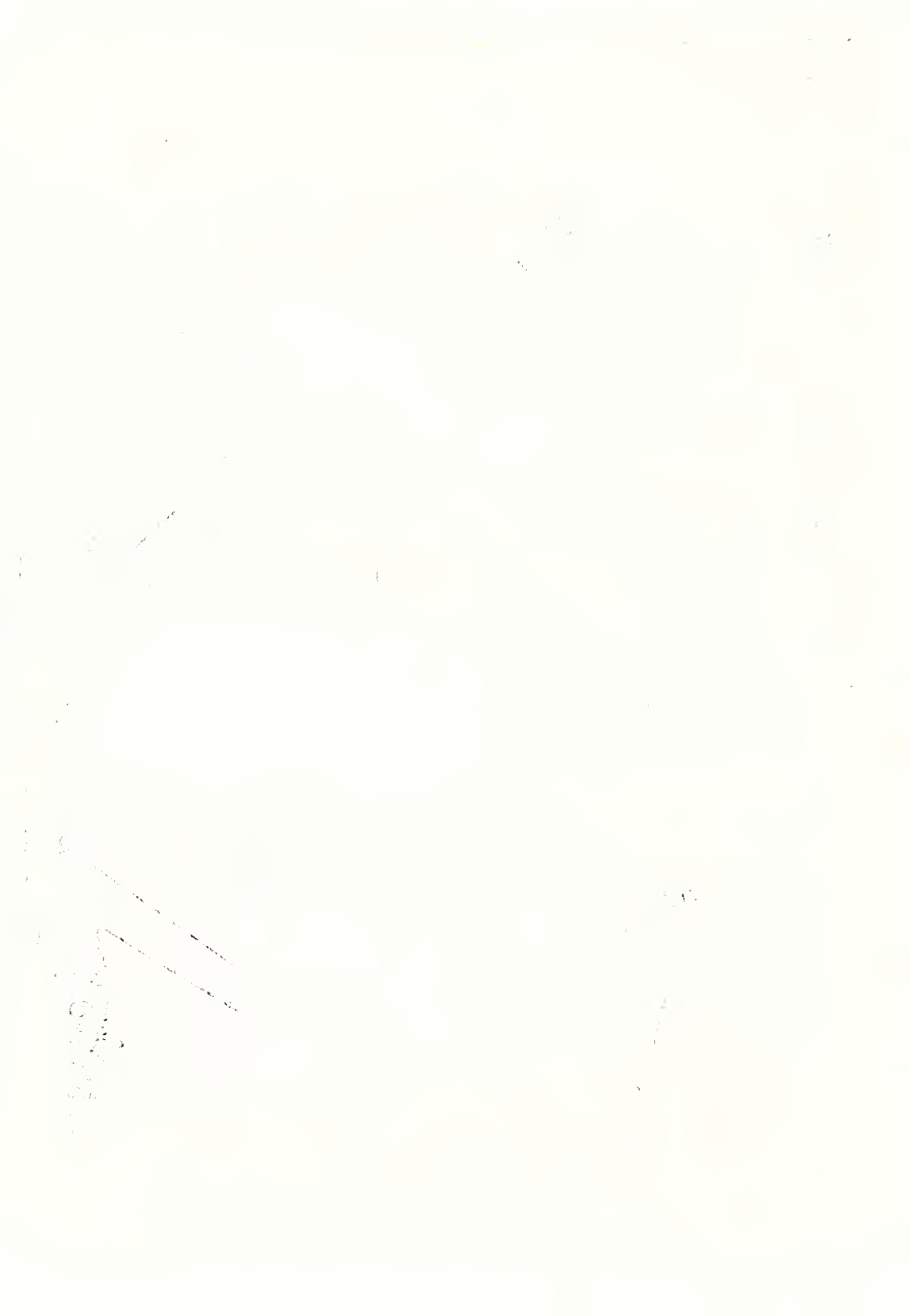
blood/gas 2.3/1

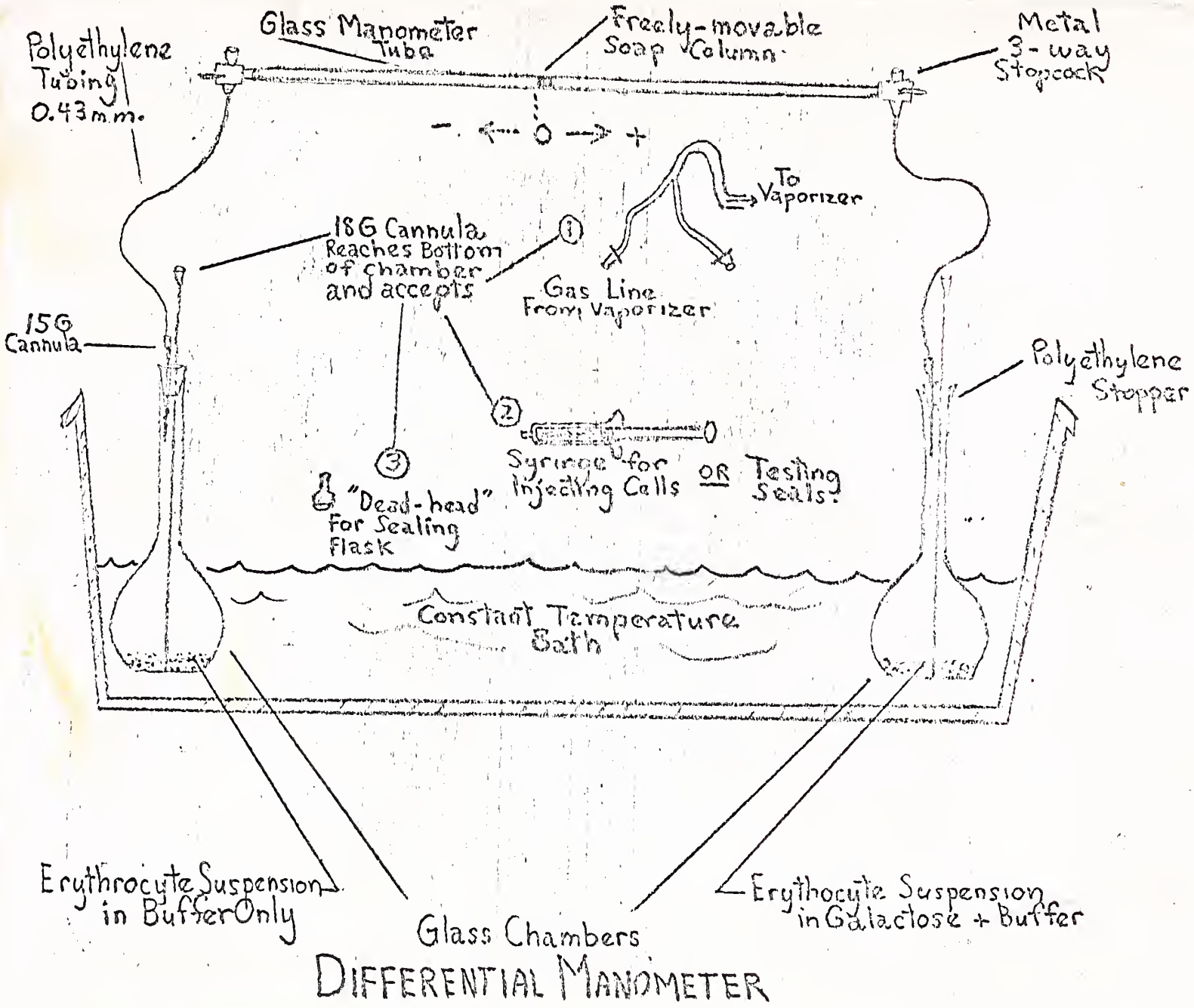
fat/blood 60/1

brain/blood 2.6/1

Table 2.

Hct.	#1	#2	#3	#4
orig.	92.1	92.2	93.9	94.1
5'	57.3	78.4	59.2	81.3
60'	64.6	77.4	67.8	79.7
120'	74.1	76.8	74.8	79.3



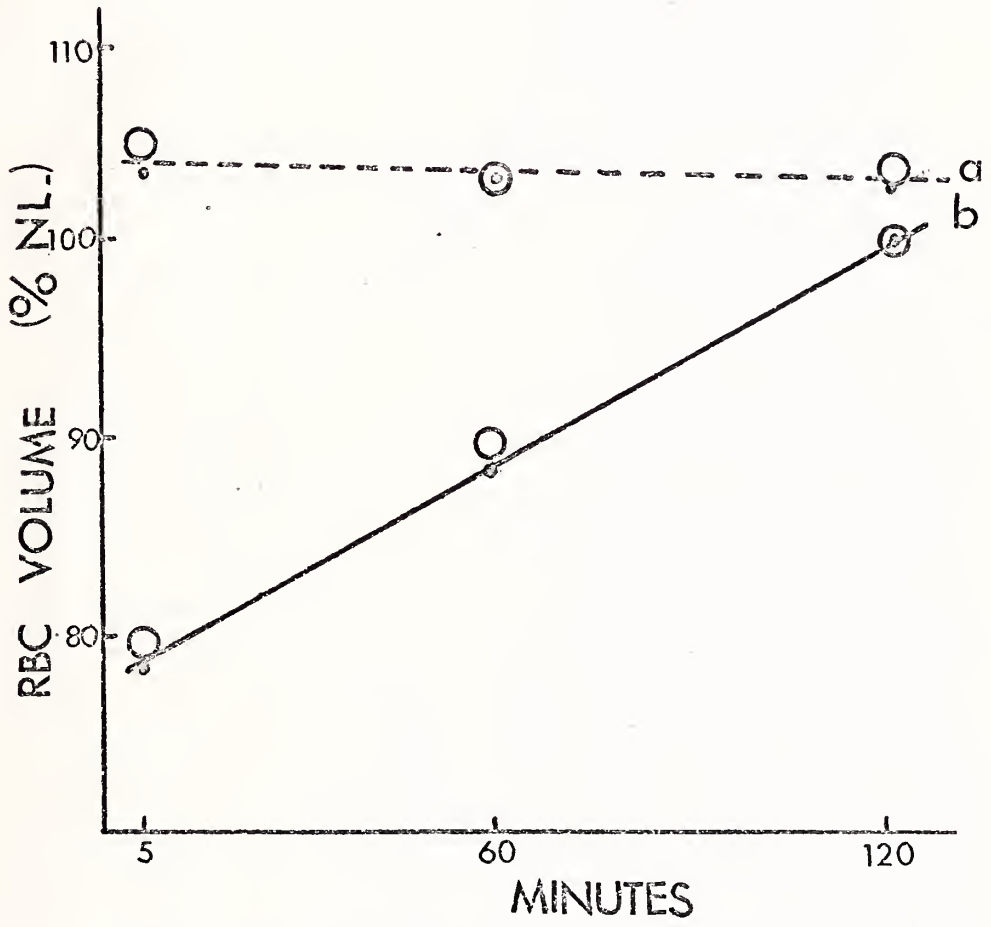


DIFFERENTIAL MANOMETER

Figure 1.

Figure 2.

Changes in RBC Volume During
Incubation and Exposure to Halothane.



o Not exposed to hal. - - - - - Treated with buffer.
• Exposed to halothane. ———— " " galactose.

Figure 3.

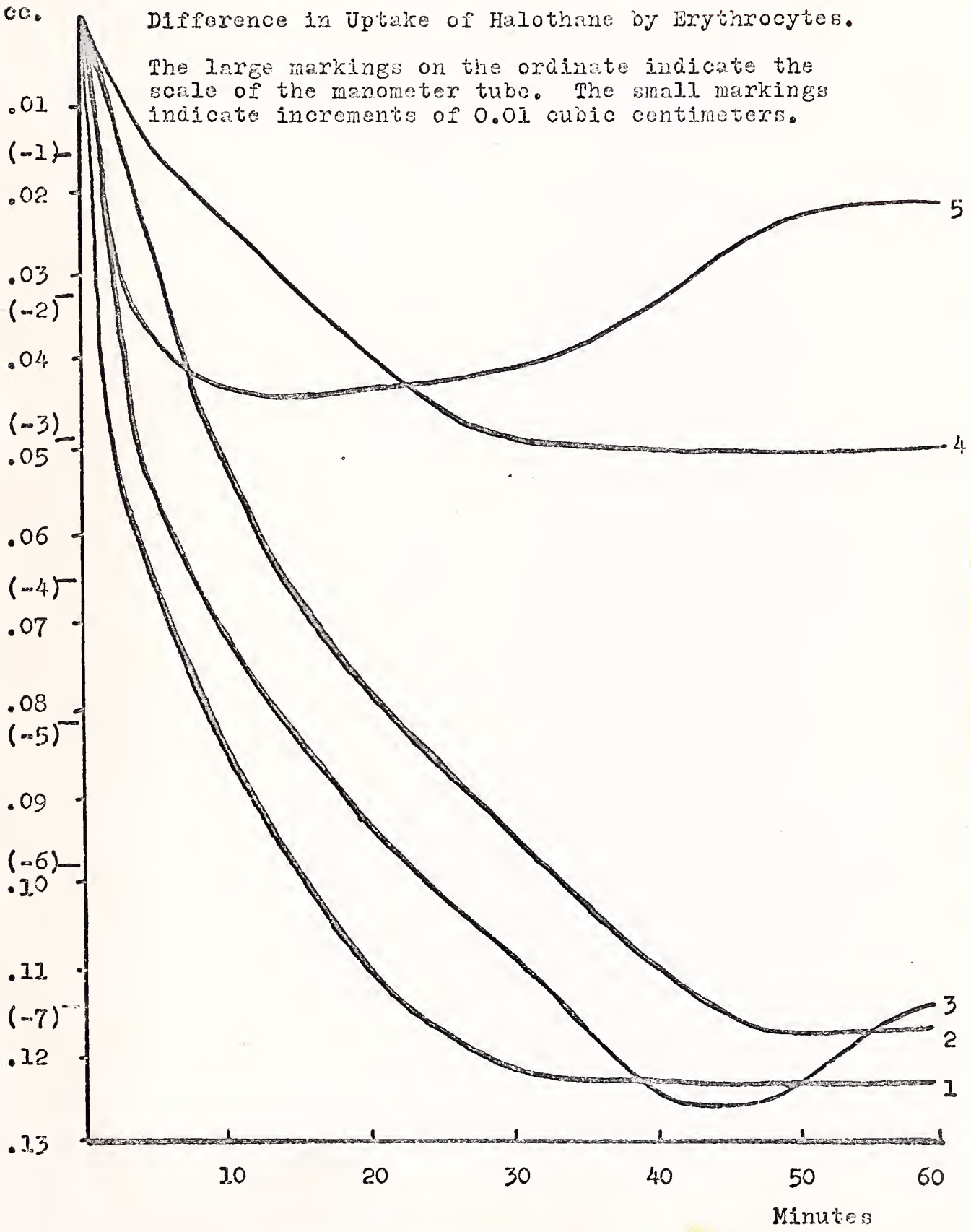
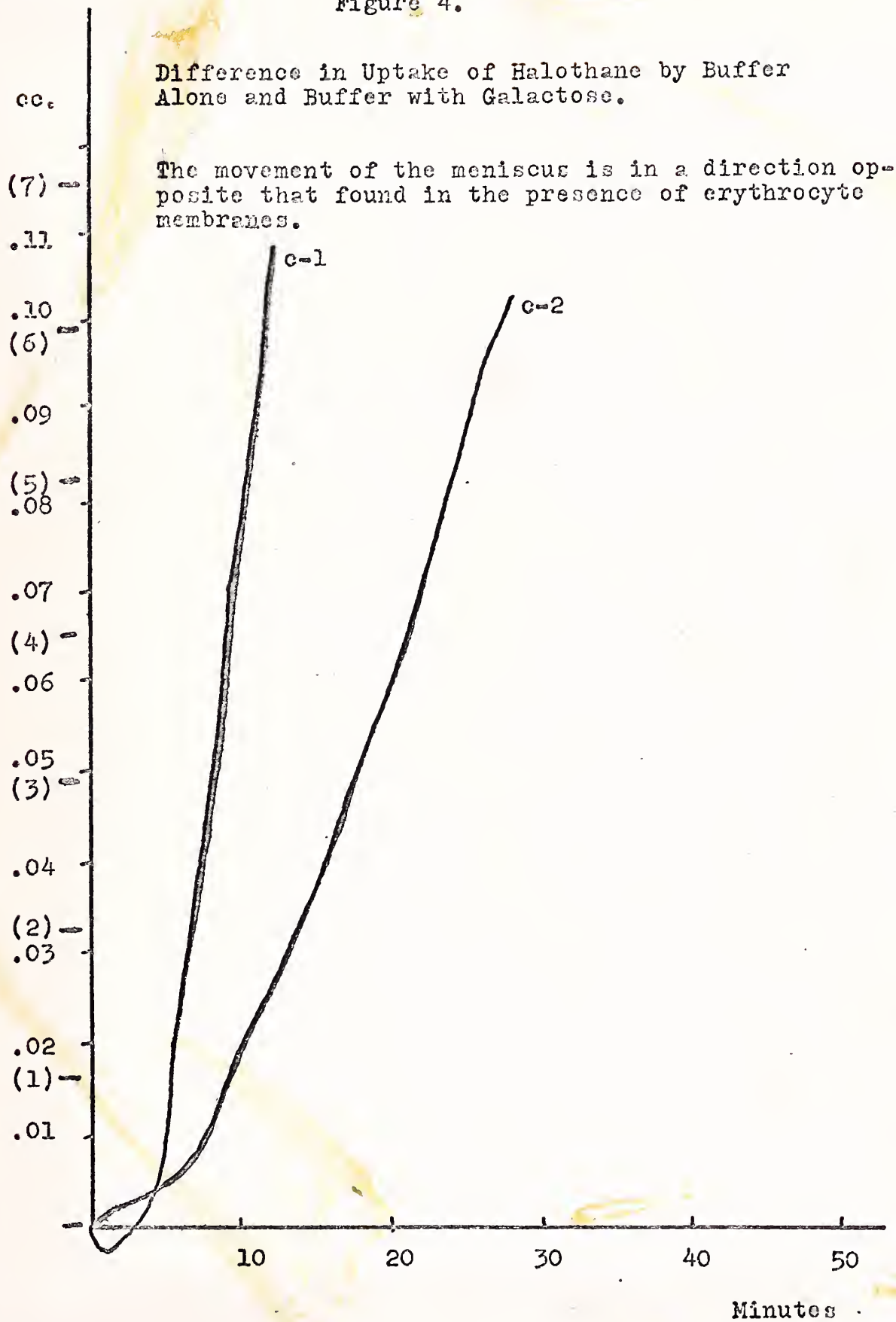

cc.
Difference in Uptake of Halothane by Erythrocytes.
The large markings on the ordinate indicate the scale of the manometer tube. The small markings indicate increments of 0.01 cubic centimeters.
.01
(-1)
.02
.03
(-2)
.04
(-3)
.05
.06
(-4)
.07
.08
(-5)
.09
(-6)
.10
.11
(-7)
.12
.13
5
4
3
2
1
10
20
30
40
50
60
Minutes

Figure 4.

Difference in Uptake of Halothane by Buffer Alone and Buffer with Galactose.

The movement of the meniscus is in a direction opposite that found in the presence of erythrocyte membranes.



Appendix 1.

I. Preparation of Sørenson's Phosphate Buffer (9)

Stock Solution A (Fifteenth molar KH_2PO_4)

Dissolve 9.078 gm. monobasic potassium phosphate in exactly 1000 ml. distilled H_2O free of chlorine and sulfates.

Stock Solution B (Fifteenth molar Na_2HPO_4)

Dissolve 9.472 gm. anhydrous dibasic sodium phosphate in exactly 1000 ml. distilled H_2O free of chlorine and sulfates.

(Modified from the original formula which specified 11.876 gm. dibasic sodium phosphate with 2 mols water of hydration added to 1000 ml. H_2O.)

Mixture of 2 parts Solution A with 8 parts Solution B yields phosphate buffer of pH 7.381

II. Preparation of Galactose-Phosphate Buffer Solution

Dissolve 6.441 gm d-Galactose in Sørenson's Buffer and dilute to 25 ml. with the same.

All chemicals used were analytic grade. Only ion-free distilled water was used for solutions. Glassware was chemically clean. Stock solutions for phosphate buffer were used up to six months after the date of preparation. Mixed buffer was discarded after one week. Galactose-phosphate buffer solution was discarded after one week.

Calibration of the Manometer Tube

Originally the manometer tube was marked in units of length (tenths of centimeters.) Calibration of the volume markings of the tube was performed by injection of fluid from a syringe marked in eighty divisions per cubic centimeter. The increments in volume per centimeter marking are presented below and show no variation in diameter of the tube, within limits of accuracy.

Manometer mark (cm.)	Volume contained (80ths of a cc.) Determination #		
	1	2	3
3	0.0	0.0	0.0
4	1.5	1.0	1.0
5	3.0	2.0	2.5
6	4.0	4.0	4.0
7	5.0	5.0	5.0
8	6.0	6.0	6.5
9	7.5	7.5	8.0
10	9.0	9.0	9.0
11	11.0	10.0	10.5
12	12.5	11.5	12.0
13	13.5	12.5	13.0
14	15.0	14.0	14.0
15	16.5	15.5	15.5
16	18.0	17.0	17.0
17	19.0	18.0	18.5
18	20.5	19.5	19.5
19	22.0	21.0	21.0
20	23.0	22.0	22.0
21	24.0	23.5	23.5
22	26.0	25.0	25.0
23	27.0	26.0	26.0
24	28.0	27.5	27.5
25	29.5	29.0	29.0
26	31.0	30.0	30.0
27	32.0	31.5	31.5
28	33.0	32.5	32.5
29	34.0	34.0	34.0
30	35.0	35.5	35.5

Thus there are 35.33/80 cc. per 27.0 cm. length, and one cm. contains 0.0164 cubic centimeters. Experimental readings were taken on the centimeter scale and translated to cubic centimeters by the formula:

cubic centimeters = (centimeter reading - 3) x 0.0164

Appendix 3.

Hematocrit Determinations

(Each experimental value listed is the mean of four or more simultaneous determinations.)

	Time (min.)	Experiment 1	2	3	4	5	Mean
Galactose Added	0	92.9	91.8	92.2	91.6	91.9	92.1
	5	56.9	58.6	55.8	57.8	57.6	57.3
	60	62.5	62.4	63.7	67.8	66.6	64.6
	120*	72.8	73.6	74.2	75.1	74.7	74.1
Buffer Only Added	0	92.6	91.8	92.6	91.8	92.0	92.2
	5	76.8	77.0	79.7	79.5	79.0	78.4
	60	76.7	75.4	78.0	78.0	78.9	77.4
	120*	77.4	76.7	76.9	76.1	76.8	76.8
Galactose Added	0	93.5	94.0	94.2			93.9
	5	56.9	60.3	60.4			59.2
	60	66.3	70.8	66.4			67.8
	120#	70.7	77.8	75.8			74.8
Buffer Only Added	0	93.7	94.1	94.6			94.1
	5	79.1	82.8	82.0			81.3
	60	78.1	80.8	80.1			79.7
	120#	78.8	80.3	78.9			79.3

* Exposed to halothane during the interval t(60 → 120).

\# Not exposed to halothane.

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